

Glucosylceramide synthesis and synthase expression protect against ceramide-induced stress

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Abstract Ceramides (Cers), critical for epidermal barrier function, also can inhibit keratinocyte proliferation, while glucosylceramides (GlcCers) exert pro-mitogenic effects. Since alterations in Cer-to-GlcCer ratios appear to modulate cellular growth versus apoptosis, we assessed whether keratinocytes up-regulate GlcCer synthesis as a protective mechanism against Cer-induced stress. Exogenous sphingomyelinase (SMase) treatment of cultured human keratinocytes (CHK) initially decreased proliferation and cellular sphingomyelin (50–60% decrease; $P < 0.001$), and increased Cer levels (6.1- to 6.8-fold; $P < 0.001$). Proliferation recovered to normal rates by 24 h, in parallel with increased cellular GlcCer. Both GlcCer synthesis and GlcCer synthase activity increased significantly by 8 h following SMase (8.2- and 2.4-fold, respectively; $P < 0.01$ each vs. control), attributed to antecedent increases in GlcCer synthase mRNA and protein expression. Further evidence that GlcCer production is responsible for normalized CHK proliferation includes: *a*) attenuation of SMase-induced inhibition of proliferation by exogenous GlcCer; and *b*) enhancement of the SMase effect in cells cotreated with the GlcCer synthase inhibitor, PDMP (D-threo-1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol). Finally, although proliferation in immortalized, nontransformed keratinocytes (HaCaT) also was inhibited by SMase, HaCaT cells that overexpress GlcCer synthase were resistant to this effect. **Thus, SMase-induced stress initiates a response in keratinocytes that includes upregulation of GlcCer synthesis which may protect against the deleterious effects of excess Cer.**—Uchida, Y., S. Murata, M. Schmuth, M. J. Behne, J. Deuk Lee, S. Ichikawa, P. M. Elias, Y. Hirabayashi, and W. M. Holleran. **Glucosylceramide synthesis and synthase expression protect against ceramide-induced stress.** *J. Lipid Res.* 2002. 43: 1293–1302.

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The epidermis of terrestrial mammals generates large quantities of ceramides (Cers) largely destined for the ex-

tracellular domains of the outermost epidermal layer, the stratum corneum, where they are critical components of the permeability barrier (1, 2). In contrast, additional studies implicate Cer generation in the induction of cellular stress responses, including accelerated differentiation, apoptosis, and senescence (3–6). In an established stress model, increased Cer can result from activation of endogenous sphingomyelinase (SMase) following several types of cellular stress, such as γ irradiation, heat shock, and inflammation, leading to hydrolysis of sphingomyelin (SM) to Cer (7–10). Similarly, exogenous SMase induces increased Cer levels, resulting in cell-specific responses including apoptosis and differentiation (11–14). Finally, increased de novo Cer synthesis from activation of either Cer synthase (15, 16) and/or serine palmitoyltransferase (17–19) can induce apoptosis in some cell types following exposure to stress. Cer generated by any of these mechanisms, in turn, appear to signal divergent downstream pathways, including activation of either a Cer-activated kinase, a cytosolic Cer-activated protein phosphatase, protein kinase C-zeta, and/or NF- κ B [(20–25); reviewed in (14, 26)].

In contrast to the growth-inhibitory effects of Cer, glucosylceramide (GlcCer) exert pro-mitogenic properties in a variety of tissues and cell types (27–30). For example, treatment of MDCK cells with a β -glucocerebrosidase inhibitor simultaneously increases cellular GlcCer levels and stimulates cellular proliferation (28). Increased endogenous GlcCer also may account, in part, for the organomegaly associated with β -glucocerebrosidase deficiency (Gaucher disease) (31). Furthermore, administration of exogenous GlcCer directly into either liver (27) or skin (29, 30) stimulates growth. These studies suggest either: 1) that Cer and GlcCer exert opposing effects on cellular

Abbreviations: Cer, ceramide; CHK, cultured human keratinocyte; GlcCer, glucosylceramide; HPTLC, high-performance thin-layer chromatography; SM, sphingomyelin; SMase, sphingomyelinase.

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growth; or 2) that GlcCer synthesis protects against the negative consequences of increased Cer. Accordingly, Hirabayashi et al. showed that exogenous SMase-induced increases in cellular Cer inhibit proliferation in UDP-glucose: Cer β -D-glucosyltransferase (GlcT-1 or GlcCer synthase)-deficient GM-95 cells, but not in GlcCer synthase-replete B16 melanoma cells (32). Similarly, Cabot et al. showed that conversion of Cer to GlcCer confers resistance to chemotherapy-induced cytotoxicity, i.e., anthracycline-sensitive MCF-7 cells became resistant when induced to overexpress GlcCer synthase (33). Recently, we showed that GlcCer attenuates the growth inhibitory effect of Cer in cultured human keratinocytes (CHK) (Y. Uchida and W. Holleran, unpublished observations). Yet, overexpression of GlcCer synthase in Jurkat cells failed to protect cells from apoptosis induced by CD95, etoposide, or γ irradiation (34).

Thus, a balance of Cer versus GlcCer appears to be critical for maintaining cellular homeostasis; i.e., between apoptosis/cytotoxicity versus proliferation/cell survival in a variety of cell types. To further explore whether diversion of Cer into GlcCer is a general, protective pathway, with important implications for cutaneous and extracutaneous tissue homeostasis, we investigated cellular responses to increased Cer using a well-established stress model, i.e., treatment of CHK with exogenous neutral SMase. SM levels decreased while Cer levels increased in keratinocytes following exposure to exogenous bacterial SMase, changes that correlated with inhibition of keratinocyte proliferation. Yet, DNA synthesis recovered concurrent with: *a*) increased GlcCer levels; *b*) increased GlcCer synthase expression; and *c*) overexpression of GlcCer synthase in cells exposed to SMase-induced stress. These results are consistent with the hypothesis that the Cer-to-GlcCer synthetic pathway helps to protect keratinocytes from Cer-induced stress.

MATERIALS AND METHODS

Materials

Bacterial SMase (both recombinant and from *Staphylococcus aureus*) were purchased from Funakoshi (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. Conduritol B-epoxide (CBE) and *N*-octanoylsphingosine were obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada) and Matreya Inc. (Philadelphia, PA), respectively. β -D-glucosyl-(*N*-acetyl)-sphingosine (GlcC2Cer) was prepared by acetylation of glucosylsphingosine (Sigma) with acetic anhydride and subsequent purification. Other lipids were from Sigma. D-threo-PDMP (D-threo-1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol) was a gift from Dr. Norman Radin (*Emeritus*; Mental Health Research Institute, University of Michigan, MI). [Methyl- 3 H] thymidine (85 Ci/mmole), L-[3 H]serine (147 Ci/mmole), [3 H]galactose (40–60 Ci/mmole), and [3 H]UDP-glucose (60 Ci/mmole) were from American Radiolabeled Chemical Inc. (St. Louis, MO) and Amersham Life Sciences (Arlington Heights, IL). High-performance thin-layer chromatography (HPTLC) plates (Silica Gel 60) were purchased from Merck (Darmstadt, Germany).

Cell culture

Normal human keratinocytes were isolated from human neonatal foreskins by the method of Pittelkow and Scott (35), as modified (36). Second-passage cells were grown in keratinocyte growth medium, supplemented with bovine epidermal growth factor, bovine pituitary extract, insulin, hydrocortisone, and 0.07 mM calcium (KGM; Clonetics, San Diego, CA; or Cascade Biologics, Portland, OR) to 80–95% confluence. Immortalized, non-transformed, low-passage number HaCaT cells, derived from human epidermis, were a gift from Dr. N. Fusenig (Heidelberg, Germany). The cultures were maintained at 37°C under 5% CO₂ in air.

Retroviral gene transduction

GlcCer synthase (1.2 kB): pLNCX2 Retroviral vector or empty vector, were transfected into PT-67 cell-packaging cell line. HaCaT cells were incubated with virus-containing cultured cell supernatant in the presence of 8 μ g/ml of polybrene 32°C for 24 h. Selection of transduced cells was performed following the addition of G418 (0.8 mg/ml).

Cellular proliferation

Cells were seeded in twelve or twenty four-well multiwell plates, cultured to 80–100% confluence, and then treated with SMase and/or GlcC2Cer. GlcC2Cer was complexed with fatty-acid-free BSA (1:1 molar ratio) prior to addition to the medium. Aqueous soluble SMase was dissolved directly in the medium. Stock solutions were prepared immediately prior to use. Total cellular DNA was assayed by the method of Labarca and Paigan (37). Keratinocyte growth was assessed by measuring [methyl- 3 H]thymidine incorporation into DNA. After appropriate incubations with the test compounds, cells were incubated with 1 μ Ci/ml of [3 H]thymidine for 1 h at 37°C, and the quantity of label in TCA-precipitable macromolecules was determined by liquid scintillation spectroscopy. DNA synthesis was expressed as [3 H]thymidine incorporated per microgram DNA.

Lipid fractionation and quantitation

Lipid extracts were prepared from cellular homogenates by the method of Bligh and Dyer (38). Separation of individual lipid species was achieved by HPTLC and quantitated by densitometry as previously described (1). GlcCers and Cers were separated first using chloroform-methanol-water (40:10:1, v/v/v) to 2.0 cm and to 5.0 cm, then chloroform-methanol-acetic acid (94:4:1, v/v) to 8.5 cm, followed by n-hexane-diethyl acetic acid (65:35:1, v/v/v) to the top of the plate. SM was separated using chloroform-methanol-acetic acid-water (50:30:8:4, v/v/v/v).

To assess the fate of preformed sphingolipids, keratinocytes were incubated with [3 H]serine (1 μ Ci/ml) for 2 days and washed with phosphate-buffered saline. Cells were incubated in fresh medium with or without SMase. To then examine de novo lipid synthesis, cells were cultured with SMase and then incubated with [3 H]serine (1.5 μ Ci/ml) or [3 H]galactose (2.0 μ Ci/ml) during the final 3 h of incubation. Lipids were extracted and analyzed as described above. Radioactivity in each lipid fraction was determined using a Beckman LS-1800 scintillation counter. Lipid synthesis was expressed as cpm per mg DNA.

Glucosylceramide synthase assay

The assay for GlcCer synthase activity was performed according to the method of Shukla and Radin (39) with modification (40). Keratinocyte homogenates (150–200 μ g protein) were incubated with 100 μ M [3 H]UDP-glucose (Spec. Act., 20,000 dpm/nmole), 1 mM dithiothreitol, 2 mM EDTA, 10 mM MgCl₂, 2.5 mM NADH, 20 mM Tris-HCl buffer, pH 7.4, and liposomal substrate (42.5 μ g *N*-octanoylsphingosine, 250 μ g dipalmitoylphosphatidylcholine,

and 50 μ g sulfatide) in a total volume of 0.2 ml at 32°C (60 min). The reaction was terminated by adding 500 μ l of chloroform-methanol (1:1, v/v). The mixture was centrifuged for 5 min at 3,000 rpm. The lower organic phase was washed twice with water saturated with chloroform-methanol, and the incorporation determined by liquid scintillation spectrometry, as above.

Reverse transcriptase polymerase chain reaction

Each DNase I-treated total RNA sample was prepared from cells using the RN-easy mini kit and RNase-free DNase (Qiagen, Valencia, CA). cDNA was reverse transcribed from DNase I-treated total RNA with random hexamer using the SuperScript Preamplification System for First Strand cDNA Synthesis (GIBCO-BRL, Grand Island, MD). An aliquot of the first strand cDNA was used to PCR amplify both the 400 bp GlcCer synthase and the 801 bp GAPDH gene fragments using *Taq* DNA polymerase (Qiagen). The following sets of primer pairs were used: GlcCer synthase-F1 (derived from GlcT-1) (42): 144, 5'-CAAGCTCCCAGGTGTCTCTCT-3', 165; GlcCer synthase-R1 (derived from GlcT-1): 543, 5'-AGCAAAGCCCTGTCTGTCTGCT-3', 522; GPDH-F1 (derived from GAPDH): 317, 5'-AGAGGGCTGGGGCTCATTTCGA-3', 338; GPDH-R1 (derived from GAPDH): 1117, 5'-AGGAGGGGAGATTCAGTGTGGT-3', 1096. Confirmation of PCR products was performed following electrophoresis (1.5% of agarose) with the size of resultant products compared with a coamplified control template(s). In addition, fragments obtained by *Hind*III restriction endonuclease digestion were compared with those from control templates.

Riboprobe preparation

The 400 bp GlcT-1 fragment was amplified from human GlcCer synthase (GlcT-1) cDNA (pCG-2) (41) using GlcCer synthase-F1 and GlcCer synthase-R1 primers. The PCR product was ligated with T7 promoter using the Lig'nScribe Kit (Ambion, Austin TX) and subjected to *in vitro* transcription to yield digoxigenin-labeled RNA probe using an RNA labeling kit (Roche Molecular Biochemicals, Mannheim, Germany).

Northern blotting analysis

Poly(A)⁺ RNA was prepared as described previously (42, 43). Three micrograms of poly(A)⁺ RNA were electrophoresed on a 1.5% agarose gel containing 0.4 M formaldehyde, followed by capillary transfer and UV cross-linking to a positively charged nylon filter (Roche Molecular Biochemicals). The membrane was hybridized with both the digoxigenin-labeled anti-sense GlcCer synthase probe in Dig Easy Hyb solution (Roche) and the Dig-labeled actin RNA probe (Roche). The membrane was washed in 2 \times SSC containing 0.1% SDS, and then in 0.1 \times SSC containing 0.1% SDS. Detection was performed by addition of CSPD ready-to-use solution (Roche) to the membrane, followed by exposure to X-ray film and development.

Western immunoblot analysis

Western blot analysis was performed as described previously (42, 44). Briefly, keratinocytes were solubilized with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and protease inhibitors (CompeteTM, Roche Biochemical) and centrifuged to remove cell debris. Supernatant in sample buffer (60 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 5% β -mercaptoethanol and 0.005% bromophenol blue) was resolved by electrophoresis on 12% SDS-polyacrylamide gel. Resultant bands were electrophoretically transferred to polyvinylidene difluoride membranes (Biorad, Hercules, CA), probed with anti-GlcCer synthase antiserum (kind gift of Dr. Richard Pagano, Rochester MN), and detected using an enhanced chemiluminescence system (NEN, Wilmington, DE).

Statistical analyses

Statistical analyses were performed using an unpaired Student *t*-test.

RESULTS

Exogenous SMase inhibits DNA synthesis in CHK

Previous studies have shown that either exogenous Cer administration or increased endogenous Cer resulting from inhibition of GlcCer synthase, results in a decline in CHK proliferation that reaches its nadir between 18 and 24 h (6) (Y. Uchida and W. Holleran, unpublished observations). To ascertain whether Cer mobilized from the membrane pool of SM exerts similar effects, we first measured incorporation of [³H]thymidine into DNA (DNA synthesis) after addition of exogenous SMase. Between SMase concentrations of 0.01–0.1 U/ml, no direct toxicity to cells was noted, i.e., trypan blue uptake remained unchanged during incubation period (data not shown). Yet, DNA synthesis decreased significantly 1 to 3 h following SMase treatment of CHK, returning toward normal by 8 h and thereafter (Fig. 1; *P* < 0.001, at 1, 2, and 3 h, respectively). These results indicate that SMase treatment causes a rapid decrease in CHK proliferation.

SMase-induced changes in proliferation parallel changes in cellular sphingolipid content

To assess whether the effects of SMase could be mediated by changes in cellular sphingolipid content, we next measured cellular SM, Cer, and GlcCer levels before and after SMase exposure, as well as in vehicle-treated controls. Whereas SM content decreased precipitously between 1 and 8 h following SMase treatment (i.e., >50–60% decrease) (Fig. 2A), levels of other phospholipids, including phosphatidylcholine, remained unchanged (data

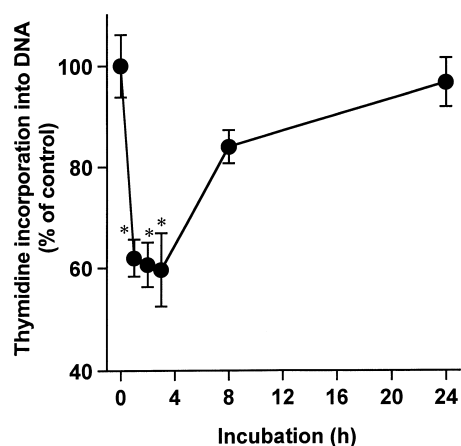


Fig. 1. Exogenous sphingomyelinase (SMase) reversibly inhibits cultured human keratinocyte proliferation. Human keratinocytes (second passage), cultured in serum-free KGM, were treated with SMase (0.05 U/ml). Incorporation of [³H]thymidine (during the final hour of incubation) was measured, and results are presented as percent of vehicle control. Values represent the mean \pm SEM for *n* = 6 determinations; **P* < 0.001 versus vehicle-treated control cells.

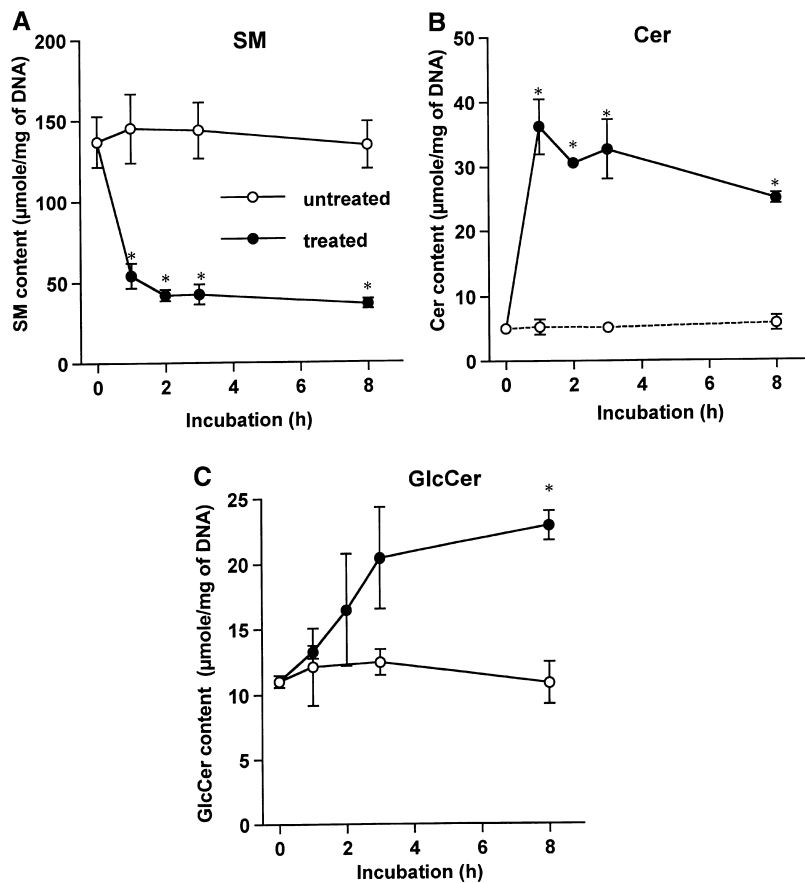


Fig. 2. SMase alters ceramide (Cer), glucosylceramide (GlcCer), and sphingomyelin (SM) content in cultured human keratinocytes (CHKs). Human keratinocytes were incubated with vehicle (untreated; open circles) or SMase (0.05 U/ml) (treated; closed circles) for up to 8 h, as indicated. Total lipids were extracted and isolated by high-performance thin-layer chromatography (HPTLC). Individual sphingolipid species, i.e., SM (A), Cer (B), and GlcCer (C) were quantitated as described in Materials and Methods. Values represent the mean \pm SEM for $n = 3$ determinations. * $P < 0.001$ versus vehicle-control for corresponding time point.

not shown). Conversely, Cer levels increased rapidly (i.e., >6-fold; $P < 0.001$) over vehicle-treated controls between 1 and 3 h, remaining elevated up to 8 h (i.e., 4.4-fold; $P < 0.001$) (Fig. 2B). In contrast, GlcCer levels increased more slowly over time, reaching a 2.1-fold increase 8 h after SMase exposure (Fig. 2C). These results show that the rebound in CHK proliferation parallels divergent changes in SM, Cer, and GlcCer content.

SMase treatment activates GlcCer synthesis

We next assessed whether the rebound in DNA synthesis and increase in GlcCer content that occur after SMase treatment are linked to increased GlcCer synthesis. Two approaches were employed; first, we assessed the fate of prelabeled ($[^3\text{H}]$ serine) sphingolipids. While the level of labeled SM decreased significantly, as expected at 8 h following SMase (i.e., to 51.6% of untreated controls), the levels of labeled Cer and GlcCer both increased markedly (i.e., to 822% and 573%, respectively; $P < 0.01$ in SMase-treated vs. control cells) (Fig. 3A). The increase in labeled Cer plus GlcCer (i.e., +56,100 cpm/mg of DNA) exceeded the decrease in labeled SM (i.e., -18,500 cpm/mg of DNA), consistent not only with diminution of the preformed SM pool but also increased de novo production of both Cer and GlcCer (c.f., Figs. 2A–C).

Next, we investigated the effects of SMase treatment on de novo synthesis of sphingolipids in CHK. When cells were incubated with $[^3\text{H}]$ serine during SMase treatment,

incorporation into SM decreased (i.e., to 35% of control cell values) (Fig. 3B); conversely, incorporation into both total Cer and total GlcCer pools increased significantly (i.e., 2.7-fold and 1.8-fold, respectively), rates that together, again exceeded the decrease in SM labeling. These studies show that production of both Cer and GlcCer increase after SMase treatment.

GlcCer synthase expression increases following SMase treatment

To investigate whether the increase in GlcCer generation after SMase treatment is due to activation of GlcCer synthase, we next measured the enzyme activity in cell homogenates of SMase-treated versus control CHK. Although numerous epidermal lipid-synthetic enzymes are activated following barrier perturbation (1, 45–47), GlcCer synthase activity does not change (40). Hence, the constitutive level of GlcCer activity in the epidermis appears to be sufficient to accommodate the requirements for increased extracellular GlcCer/Cer following barrier perturbations. Yet, consistent with the lipid synthesis data presented above, GlcCer synthase activity increased significantly over time following SMase-induced stress to CHK (Fig. 4). To address whether the SMase-induced increase in GlcCer synthase activity is regulated at the transcriptional level, we next examined GlcCer synthase mRNA levels after treatment. A significant increase in GlcCer synthase mRNA levels (i.e., greater than 2-fold over controls)

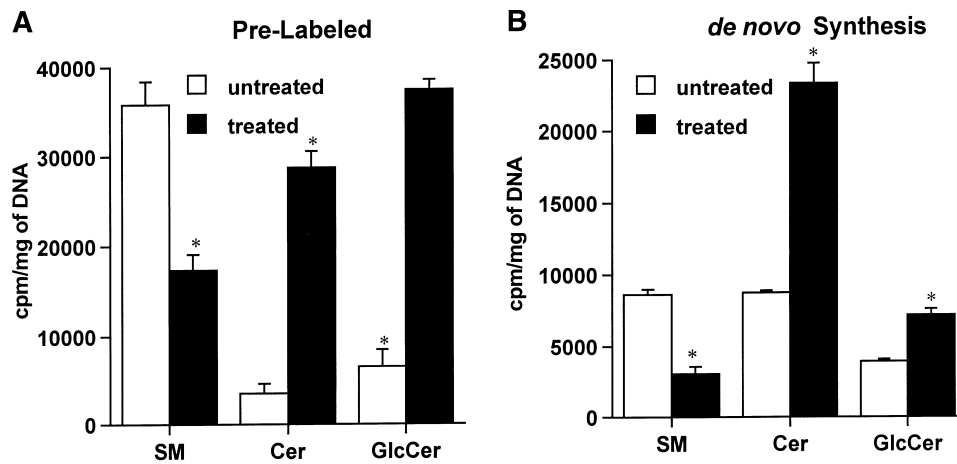


Fig. 3. SMase alters Cer, GlcCer, and SM levels in CHKs. **A:** Human keratinocytes were pre-labeled with [^3H]serine for 2 days. Cells then were rinsed and incubated with vehicle (open bars) or SMase (0.05 U/ml) in culture medium for 8 h. Total lipids were extracted, and individual sphingolipids; i.e., SM, Cer, and GlcCer were isolated by HPTLC and incorporated cpm determined, as described in Materials and Methods. **B:** de novo Cer and GlcCer synthesis. Cells were treated with SMase or vehicle for 8 h and [^3H]serine (during the final three h of incubation). Lipids were isolated by HPTLC as described in Materials and Methods. Values represent the mean \pm SEM for $n = 3$ determinations. * $P < 0.01$ versus vehicle-controls.

was evident at 18 h by Northern blot analysis (Fig. 5A). RT-PCR analysis also revealed corresponding increases in GlcCer synthase mRNA at both 8 and 18 h after SMase treatment (Fig. 5B; note lane changes). Finally, Western immunoblotting was performed to determine whether the increase in GlcCer synthase mRNA levels was reflected by enhanced enzyme protein levels. Indeed, protein levels increased at both 8 h and 18 h after SMase-treatment in comparison to levels in control cells at the corresponding time points (Fig. 5C). These results demonstrate that the SMase-induced increase in GlcCer production is associated with an increase in GlcCer synthase activity, which in

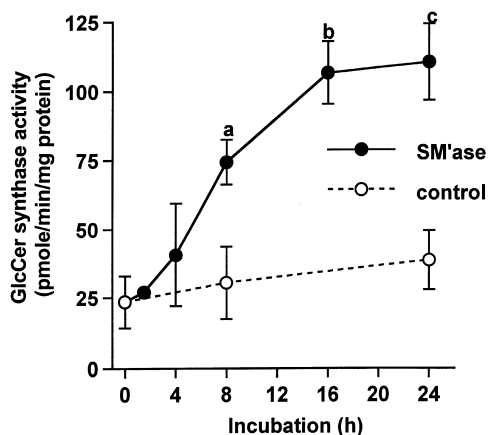


Fig. 4. SMase activates GlcCer synthase activity. Human keratinocytes were treated with SMase (closed circles) or vehicle (control; open circles) for the indicated times (0–24 h), and cell homogenates were assayed for GlcCer synthase activity. Values represent the mean \pm SEM for $n = 3$ determinations; $P < 0.02$ (a) and $P < 0.01$ (b) versus 8 h vehicle-controls; $P < 0.01$ (c) versus 24 h vehicle-control.

turn, can be attributed to increases in both enzyme mRNA and protein levels.

Increased cellular GlcCer accounts for the normalization of CHK proliferation

We next assessed directly whether the normalization of keratinocyte proliferation in the face of elevated Cer levels can be attributed to increased GlcCer production. As seen in Table 1, addition of the cell-permeable GlcC2Cer, largely attenuates the SMase-induced inhibition of CHK proliferation. In contrast, DNA synthesis rates did not differ significantly in control cultures treated with vehicle or GlcC2Cer alone, while SMase treatment alone again inhibited DNA synthesis (Table 1; c.f., Fig. 1). These results show that coadministration of exogenous GlcCer with SMase reverses the expected decrease in CHK proliferation.

In the next alternative approach to this question, we examined whether endogenous GlcCer can normalize DNA synthesis in the face of SMase treatment. CHK were co-incubated with either SMase alone, or SMase plus PDMP, an inhibitor of GlcCer synthase (48, 49). We recently demonstrated that the inhibition of DNA synthesis due to accumulation of endogenous Cer that follows PDMP treatment occurs only after a lag time of greater than 8 h (Y. Uchida and W. Holleran, unpublished observations). Here again, proliferation did not remain significantly diminished (i.e., partially recovered) at 8 h after SMase treatment (Table 2; c.f., Fig. 1). Likewise, DNA synthesis was only slightly diminished (i.e., 19.5% decreased) in PDMP-treated versus control CHK at 8 h (Table 2). In contrast, keratinocyte proliferation was inhibited significantly at 8 h when SMase and PDMP were added together (50% vs. control; $P < 0.001$). Together, these results show that provision of exogenous GlcCer normalizes DNA synthesis, while in contrast, blockade of GlcCer synthesis pre-

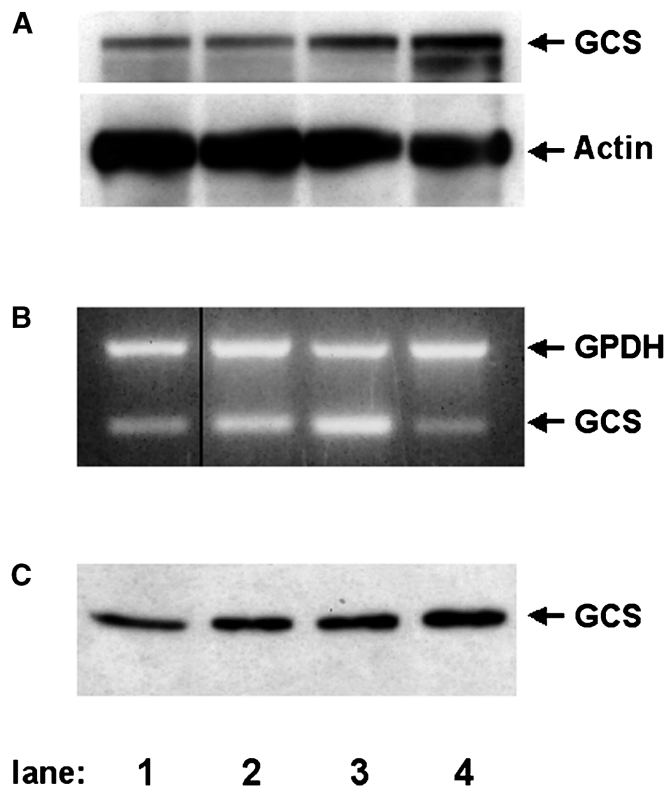


Fig. 5. SMase increases GlcCer synthase mRNA expression and protein levels. **A:** Northern-blot analysis. poly(A)⁺RNA was isolated from CHKs treated with vehicle or SMase for 8 h (lanes 1 and 3, respectively) or 18 h (lanes 2 and 4, respectively) and electrophoresed on a 1.5% agarose gel followed by hybridized with both digoxigenin-labeled anti-sense GlcCer synthase probe and the Dig-labeled actin RNA probe. **B:** RT-PCR analysis. Reverse transcription was performed using 1 μ g of DNaseI-treated total RNA from CHK treated with vehicle or SMase for 8 h (lanes 1 and 2, respectively) or 18 h (lanes 4 and 3, respectively; note change in lane position). A fraction (1/20) of the reverse transcription reaction mixture was PCR amplified for both the GlcCer synthase (400 bp) and GAPDH (801 bp) fragments (i.e., in same tube). **C:** Western-blot analysis. CHKs were incubated with vehicle or SMase for 8 h (lanes 1 and 2, respectively) or 18 h (lanes 3 and 4, respectively). Cell lysate was resolved by electrophoresis on 12% SDS-polyacrylamide gels and probed with anti-GlcCer synthase antiserum; representative blot shown.

vents the normalization of DNA synthesis rates following SMase challenge.

Overexpression of GlcCer Synthase in HaCaT keratinocytes increases resistance to SMase-induced stress

To investigate further the role of GlcCer in the rescue of keratinocytes from SMase-induced stress, we next overexpressed GlcCer synthase in HaCaT cells, a line of immortalized, nontransformed keratinocytes. To establish stable overexpression of GlcCer synthase, a GlcCer synthase gene construct (1.2 kb) was transduced using a retroviral vector system, and transfected cells then were selected by incubation with G418. GlcCer synthesis increased 2-fold in GlcCer-overexpressing cells (HaCaT-G) versus control cells (empty-vector transfected cells, HaCaT-C) (**Fig. 6A**). In contrast, neither Cer nor SM synthases were altered by overexpression of GlcCer synthase.

TABLE 1. Exogenous GlcCer reverses the inhibitory effects of SMase on DNA synthesis

Conditions	Incorporation (% of control \pm SEM)	Significance (vs. vehicle-control)
Control	100.0 \pm 6.3	—
GlcC2Cer	107.3 \pm 8.1	—
SMase	66.4 \pm 5.0	$P < 0.02$
SMase + GlcC2Cer	91.0 \pm 4.4	—

Cultured human keratinocytes were treated with C2GlcCer (2.5 μ M) and/or SMase (0.05 U/ml), or no GlcCer/SMase (vehicle-control). Incorporation of [³H]thymidine (during the final hour of incubation) was measured, and results are presented as percent of control (no added SMase). Values represent the mean \pm SEM for $n = 6$ determinations each. GlcCer, glucosylceramide; SMase, sphingomyelinase.

In addition to increased basal GlcCer levels, HaCaT-G cell also showed a significant increase in GlcCer synthesis after SMase treatment (1.4-fold vs. HaCaT-C cells) (**Fig. 6B**). Consistent with increased GlcCer synthesis in HaCaT-G versus HaCaT-C cells, GlcCer content in HaCaT-G cells was higher than in HaCaT-C cells under both basal conditions (i.e., 2.6-fold), and after SMase challenge (i.e., 1.6-fold) (**Table 3**). Concurrently, the SM and Cer content in control HaCaT-G cells decreased only 12% and 30%, respectively, versus HaCaT-C values (**Table 3**).

Finally, we assessed the proliferation of HaCaT-G versus HaCaT-C cells, both under basal conditions and following SMase treatment. Basal DNA synthesis did not differ in HaCaT-G versus HaCaT-C cells (data not shown), and SMase treatment alone again significantly inhibited DNA synthesis in HaCaT-C cells (i.e., 22% inhibition vs. control, $P < 0.01$) at 4 h (**Fig. 7**; note expanded axis), with near normalization by 8 h (14% inhibition), as in nonimmortalized CHK (c.f., **Fig. 1**). In contrast, no decrease in proliferation occurred in HaCaT-G cells following SMase treatment (**Fig. 7**). These results show that overexpression of GlcCer synthase protects keratinocytes from SMase-induced inhibitory effects.

DISCUSSION

Activation of SMase, and the subsequent increase in cellular Cer, has been implicated in a variety of cell stress responses, including exposure to exogenous TNF α , radiation, heat shock, and concurrent inflammation [reviewed

TABLE 2. PDMP plus SMase treatment significantly inhibit DNA synthesis

Conditions	Incorporation (% of control \pm SEM)	Significance
Control	100.0 \pm 5.6	—
SMase	83.5 \pm 3.8	—
PDMP	80.5 \pm 2.6	$P < 0.001$ vs. control
SMase+PDMP	50.9 \pm 2.8	$P < 0.01$ vs. PDMP

Cultured human keratinocytes, grown in serum-free KGM, were treated with SMase (0.05 U/ml) and/or PDMP (7.5 μ M) for 8 h. Incorporation of [³H]thymidine was measured, and results are presented as percent of control (0 U/ml SMase). Values represent the mean \pm SEM for $n = 6$ determinations.

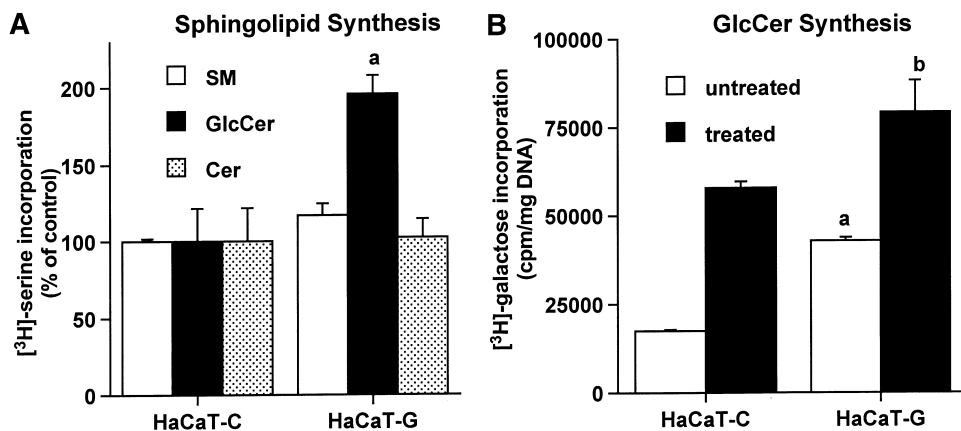


Fig. 6. GlcCer synthesis is upregulated in GlcCer synthase-overexpressing HaCaT cells. HaCaT cells were incubated with [³H] serine for 4 h (A) or with/without SMase and [³H] galactose for 4 h (B). Total lipids were extracted and each lipids were isolated by HPTLC as described in Materials and Methods and results are presented as percent of control (no added SMase). Values represent the mean \pm SEM for $n = 3$ determinations; $P < 0.001$ (a) versus HaCaT-C-untreated; $P < 0.01$ (b) versus HaCaT-C treated with SMase.

in (14, 26)]. Since the epidermis, as the outer integument of the mammalian organism, is exposed to myriad damaging and/or inflammatory stimuli, including many that alter Cer levels, we investigated here the role of GlcCer synthesis in the response of human keratinocytes to exogenous SMase, as a standard Cer stress model. As previously described in other cell types (11–14), SMase treatment acutely decreased SM, and increased Cer levels in CHKs, changes that were paralleled by a transient decline in DNA synthesis. Subsequently, GlcCer production increased in parallel with the recovery of normal cellular proliferation rates, attributable to both activation and increased production of the key enzyme of GlcCer synthesis, GlcCer synthase. These results support the hypothesis that glucosylation of Cer represents a key metabolic pathway for attenuating not only SMase-induced, but also other stressor-induced increases in Cer levels (32, 33, 50).

Whether the epidermal GlcCer production response is solely pro-mitogenic, or whether it also protects against

Cer-induced stress was addressed further. The accelerated restoration of normal CHK proliferation after SMase challenge, with coapplication of exogenous GlcCer, is consistent with our hypothesis that GlcCer is pro-mitogenic (29, 30) and therefore able to override the antiproliferative effects of exogenous Cer. In addition, a role of endogenous Cer glucosylation in regulating proliferation is further evidenced by the ability of PDMP, an inhibitor of GlcCer synthase (48, 51), to block the late rebound in DNA synthesis that occurs after SMase treatment. Whereas the addition of PDMP causes substantially less accumulation of Cer than SMase treatment (PDMP, 1.5-fold increase after 24 h), the combination of these two agents caused a synergistic decrease in CHK proliferation. These results suggest that changes in the Cer levels directly modulate proliferation and that conversion of Cer to GlcCer protects against

TABLE 3. Spingolipid content in GlcCer synthase overexpressing HaCaT keratinocytes

	Lipid Content (% of control HaCaT-C cells \pm SEM)		
	SM	Cer	GlcCer
HaCaT-C			
Vehicle	100.0 \pm 1.6	100.0 \pm 7.6	100.0 \pm 3.1
SMase	16.5 \pm 0.8 ^a	1750 \pm 8.7 ^a	215 \pm 19.0 ^a
HaCaT-G			
Vehicle	88.1 \pm 4.3	69.9 \pm 3.8 ^a	260 \pm 7.4 ^a
SMase	16.5 \pm 1.8 ^b	1333 \pm 23 ^{b,c}	357 \pm 21 ^{b,c}

HaCaT-C (vector control) or HaCaT-G (GlcCer synthase overexpressing) cells were treated with SMase (0.05 U/ml) or vehicle for 3 h. Total lipids were extracted and each lipid fraction was isolated by HPTLC as described in Materials and Methods. Results are presented as percent of vehicle-treated-control HaCaT-C cell values. Values represent the mean \pm SEM for $n = 3$ determinations.

^a $P < 0.01$, vs. vehicle-treated-HaCaT-C.

^b $P < 0.01$, vs. vehicle-treated-HaCaT-C.

^c $P < 0.01$, vs. SMase-treated-HaCaT-C.

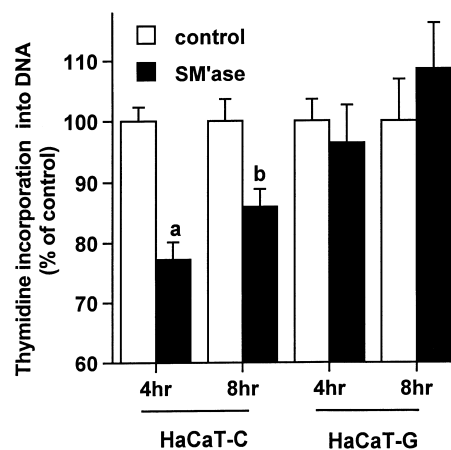


Fig. 7. GlcCer overexpression blocks inhibition of DNA synthesis following SMase. HaCaT cells were incubated with SMase or vehicle for 4 h or 8 h. Incorporation of [³H]thymidine (during the final hour of incubation) was measured, and results are presented as percent of vehicle control (note expanded ordinate scale). Values represent the mean \pm SEM for $n = 6$ determinations; $P < 0.01$ (a) and $P < 0.05$ (b) versus controls.

this Cer-induced effect. Along with the prior reports of direct mitogenic effects of GlcCer in vivo (27–30), the increased production of GlcCer that we showed here to occur during recovery from SMase-induced stress together reflect the likely importance of Cer-to-GlcCer conversion in the attenuation of Cer-induced inhibition of growth. Cabot et al. (33, 52) similarly demonstrated that the sensitivity of MCF-7 breast cancer cells to adriamycin-induced cytotoxicity is linked to levels of GlcCer synthase activity. Consistent with the protective effect of Cer glucosylation, Bieberich and colleagues recently showed that enhanced production of b-series complex gangliosides by the overexpression of sialyltransferase-II in murine neuroblastoma (F-11) cells reduced Cer-induced apoptosis (53). Thus, Cer glycosylation, to both simple and possibly complex glycosphingolipids, yields both protective and pro-mitogenic responses that warrant additional study.

Although Tepper and colleagues report no rescue from apoptosis by GlcCer synthase overexpression in Jurkat cells (34), the keratinocyte mitotic response described here is sensitive to changes in the levels of GlcCer synthase activity. Pertinently, the increased GlcCer synthesis in GlcCer synthase-overexpressing HaCaT-G keratinocytes was associated with attenuation of the SMase-induced inhibition of proliferation. These results can be interpreted in at least two ways: first, since the Cer content in SMase-treated HaCaT-G keratinocytes was significantly less than in control SMase-treated cells, and since Cer-to-GlcCer conversion is increased in HaCaT-G cells, a portion of the SMase-generated Cer could be converted to GlcCer as a protective response. Second, overexpression of GlcCer synthase could alter the overall composition of membrane sphingolipids, leading to a lower SM content in the outer leaflet of the plasma membrane, thereby making cells less susceptible to SMase. However, since the basal SM content in HaCaT-G cells (i.e., prior to SMase treatment) does not change significantly, this second interpretation seems unlikely. In either scenario, these results strongly suggest that the balance of cellular SM, Cer, and GlcCer levels regulates rates of keratinocyte growth.


It is well established that epidermal GlcCers are important precursors in the formation of stratum corneum Cer, which in turn, are essential constituents of the mammalian epidermal permeability barrier. GlcCer synthase activity is highest in the outer, more-differentiated cell layers of the epidermis (40), consistent with increased GlcCer levels during epidermal differentiation (54–56). In differentiated keratinocytes, GlcCer is primarily stored with other lipids and hydrolytic enzymes in membrane-encased lamellar bodies, the contents of which are secreted into the intercellular domains during epidermal terminal differentiation. The subsequent, downstream hydrolysis of GlcCer to Cer is a critical step in epidermal barrier formation and maintenance (57, 58). Not only does this lamellar body-dependent mechanism deliver the necessary barrier lipids to the stratum corneum but it also protects the cytosol of differentiating keratinocytes from the adverse effects of accumulated Cer by sequestering Cer in the form of a “nontoxic” precursor; i.e., as GlcCer. Moreover,

although local alterations of plasma membrane lipid composition, including hydrolysis of plasma membrane SM, occur late in differentiation, the presence of abundant GlcCer in the cytosol may protect outer epidermal cells, i.e., the granular layer, from premature apoptosis caused by release of SMase-dependent Cer. Since many of the stressful stimuli that increase epidermal cytokine levels (e.g., TNF α and IL-1 α), also have been shown to activate SM-to-Cer hydrolysis in extracutaneous tissues, tolerance to these stimuli in the outer epidermal layers may prevent unnecessary responses/reactions in deeper epidermal layers. In this context, the activation of GlcCer synthase and the subsequent formation of GlcCer may have physiological relevance for the maintenance of epidermal homeostasis in response to numerous external stressors.

The source of the Cer leading to increased GlcCer production after SMase stress remains unclear. For example, constitutive overexpression of GlcCer synthase in Jurkat cells not only increased the conversion of exogenous Cer to GlcCer but also induced de novo Cer formation under these conditions (34). In addition, the accumulation of Cer induced by γ irradiation, etoposide or exogenous bacterial SMase is not attenuated in the GlcCer synthase-overexpressing cells. Yet, we show here that keratinocyte GlcCer synthesis increased in a time-dependent manner in parallel with recovery of proliferation following SMase treatment. Thus, it is likely that, at least in keratinocytes, GlcCer synthase utilizes SM-derived Cer at later time points during the normalization of proliferation following Cer-induced stress. In addition, since de novo Cer synthesis increased in SMase-treated keratinocytes (Fig. 4), the conversion of de novo synthesized Cer-to-GlcCer also may play an important role in restoration of cellular proliferation in SMase treated CHK. However, the relative contribution of hydrolysis versus de novo synthesized Cer to the generation of GlcCer that leads to restoration of DNA synthesis remains unresolved.

To better understand the epidermal response to elevated Cer levels, we also investigated here the mechanisms by which increased GlcCer synthesis occurs in keratinocytes following SMase treatment. Previously, Abe, Radin, and Shayman reported that increased Cer levels are associated with elevated GlcCer synthase activity (59), while SMase-dependent upregulation of GlcCer synthase activity in B-16 melanoma cells is dependent on both transcriptional and posttranscriptional mechanisms (32). In the present study, GlcCer synthase mRNA levels increased in time-dependent manner following SMase treatment, changes that were accompanied by enzyme protein accumulation. In addition, the early increase in GlcCer synthesis (i.e., between 0 and 3 h) suggests that posttranslational mechanism(s) also may be involved. Moreover, the levels of GlcCer synthase mRNA in CHK are elevated both by Ca²⁺- (60) and phorbol ester-treatment (61). Although the phorbol effect is protein kinase-C-dependent (61, 62), the SMase-induced increase in GlcCer synthase mRNA appears independent of this pathway (62). Thus, although Cer levels are elevated both during CHK differentiation, including both Ca²⁺- and phorbol ester-dependent pro-

cesses, as well as in response to SMase, the operative pre- and posttranscriptional mechanisms for GlcCer synthase up-regulation by these two agents appear distinct.

Finally, the Cer-to-GlcCer conversion in cellular homeostasis may be an important rescue mechanism for a wide variety of insults. For example, we recently reported that hepatic GlcCer synthase is acutely up-regulated in Syrian hamsters in response to systemic endotoxin (LPS) treatment, with concurrent increases in hepatic and serum glycosphingolipids (63–65). In addition, serum glycosphingolipids reportedly increase in cases of acute viral hepatitis (66). Interestingly, activation of another glucosyltransferase, steroyl-glucosyltransferase, also has been reported following heat shock in both *Physarum polycepharum* and human fibroblasts (67). This increase in enzyme activity precedes both the activation of a Ca²⁺-dependent protein kinase and elevation in heat shock protein expression. Together with the present results, these varied findings suggest that increased production of specific lipid species, including both glucosylated sphingolipids and sterols, may represent a generalized response to a variety of cellular and tissue “stressors.” The extent to which these lipid response mechanisms contribute to general tissue eucrasia requires further evaluation. 

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